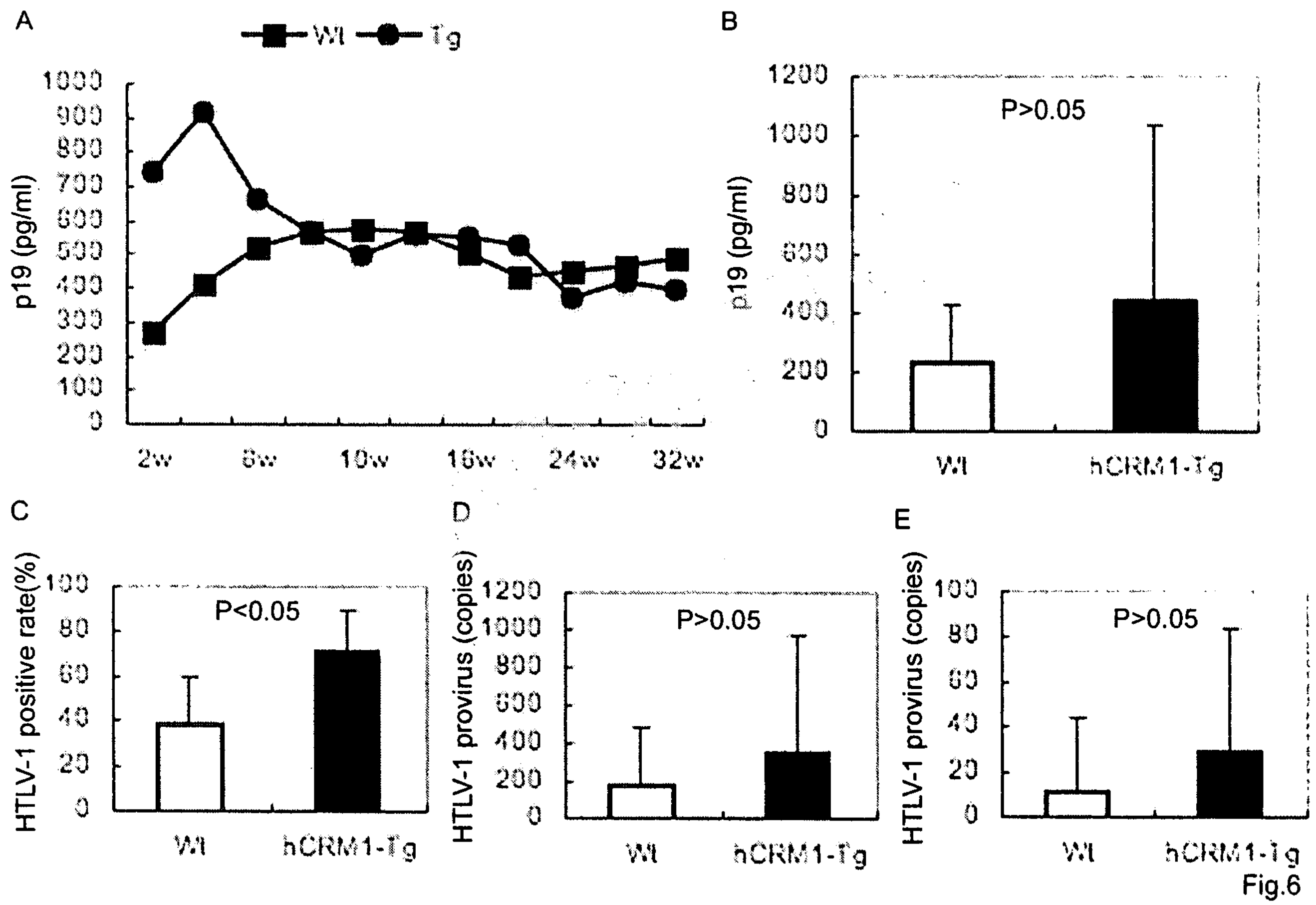

Fig.6

JVI Accepts, published online ahead of print on 7 March 2007
J. Virol. doi:10.1128/JVI.01928-06
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A novel nuclear import of Vpr promoted by importin α is crucial for HIV-1 replication in macrophages

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Running title: atypical nuclear import of Vpr in macrophages

Keywords: HIV-1 Vpr / importin α / monocyte lineage / nuclear import / viral replication

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ABSTRACT

Monocytes/macrophages are major targets of human immunodeficiency virus type 1 (HIV-1) infection. The viral pre-integration complex (PIC) of HIV-1 enters the nucleus of monocyte-derived macrophages, but very little PIC migrates into the nucleus of immature monocytes. Vpr, one of the accessory gene products of HIV-1, is essential for the nuclear import of PIC in these cells, although the role of Vpr in the entry mechanism of PIC remains to be clarified. We have shown previously that Vpr is targeted to the nuclear envelope and then transported into the nucleus by importin α alone, in an importin β-independent manner. Here we demonstrate that the nuclear import of Vpr is strongly promoted by the addition of cytoplasmic extract from macrophages, but not from monocytes, and that the nuclear import activity is lost with immunodepletion of importin α from the cytoplasmic extract. Immunoblot analysis and real-time PCR demonstrate that immature monocytes express importin α at low levels, whereas the expression of three major importin α isoforms markedly increases upon their differentiation into macrophages, indicating that the expression of importin α is required for nuclear import of Vpr. Furthermore, interaction between importin α and the N-terminal α-helical domain of Vpr is indispensable, not only for the nuclear import of Vpr, but also for HIV-1 replication in macrophages. This study suggests the possibility that the binding of Vpr to importin α, preceding a novel nuclear import process, is a potential target for therapeutic intervention.

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INTRODUCTION

Monocytes/macrophages are major targets of human immunodeficiency virus type 1 (HIV-1) and serve as a viral reservoir (12). Most tissue macrophages are permissive for the entry of macrophage-tropic viruses and they release small amounts of viral particles in asymptomatic carriers (6). In AIDS patients with opportunistic infections, macrophages occasionally produce large quantities of viral particles (40). Recent studies suggest that HIV-1 in latently infected macrophages in some lymphoreticular tissues cannot be eradicated by highly active antiretroviral therapy (HAART), and that residual cells may produce viral particles that can spread throughout the body (39). Although the extent of reverse transcription is similar in monocytes and macrophages after infection with an HIV-based vector, nuclear entry is blocked in monocytes (34). Therefore, it has been considered that active nuclear import of the viral pre-integration complex (PIC) of HIV-1 is inefficient in monocytes. However, the precise mechanisms by which the nuclear import of PIC is controlled in monocytes and differentiated macrophages are not fully understood, although this distinction is critical for the design of anti-viral strategies.

Nuclear-import processes involve the nuclear-pore complexes (NPCs) of the nuclear envelope and typically require nuclear-localization signals (NLSs). The nuclear import of basic NLS-containing proteins is mediated by specific soluble factors

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composed of two essential components, importin α and β (13). The central portion of importin α, which contains armadillo repetitive motifs, recognizes the NLS, and its N-terminal basic region, termed the importin β-binding (IBB) domain, binds to importin β (13). The ternary complex docks at the NPC and is translocated into the nucleus. Therefore, importin α acts as an adapter molecule between cargo proteins and importin β, and it is importin β that actually conveys the cargo from the cytoplasm into the nucleus. In addition, several other factors participate in this transport system, including the small GTPase Ran and its binding protein, nuclear transport factor 2 (NTF2) (32). However, there are other pathways that mediate nuclear import: transportin (transport factor of M9-containing cargo) and importin β are competent to transfer some cargo by themselves (41, 46). Moreover, it has recently been reported that importin α can migrate into the nucleus in an importin β- and Ran-independent manner (29). In addition, importin α alone can escort Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) into the nucleus without utilizing the classical importin β-dependent transport system (23).

Mammals, such as humans and mice, possess at least six importin α isoforms (importin α1/Rch1, α3/Qip1, α4/hSRP1γ, α5/NPI1, α6, and α7; 8, 21, 22, 38, 44). These isoforms can be divided into three major subfamilies according to their the amino-acid similarities: α1, α3 and α4, and α5-α7 (22). Proteins in the three groups share about 50% overall amino-acid identity. Many studies have shown that importin α isoforms differ in efficiency with respect to classical substrate-specific import (31, 45),

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and show unique expression patterns in various tissues. This suggests that importin α contributes primarily to tissue-specific nuclear transport. However, the expression patterns of importin α isoforms in human peripheral blood mononuclear cells (PBMC) are unknown.

The ability of HIV-1 to replicate in non-dividing cells such as macrophages depends on the active nuclear import of the viral PIC (4). The HIV-1 PIC contains viral proteins such as reverse transcriptase, integrase (IN), nucleocapsid (NC), Vpr, and matrix (MA, p17), in addition to viral nucleic acids (5). MA, Vpr, and IN have all been implicated in the nuclear import of PIC, although their precise roles are controversial. Both MA and IN have functional NLSs that resemble the canonical NLS of the simian virus (SV) 40 T-antigen, and both utilize the classical nuclear-import pathway that includes interaction with importins α and β (11). In contrast, despite the lack of an identifiable canonical NLS, Vpr displays karyophilic properties and it is rapidly targeted to host-cell nuclei after infection (26). Furthermore, it has been reported that the nuclear import of Vpr is mediated by an as-yet-unidentified pathway that is distinct from the classical NLS- and M9-dependent pathways (16). In this context, we have previously shown that Vpr traverses the NPC in an importin α-dependent manner (19). Vpr has also been implicated in the nuclear import of proviral DNA in macrophages (7, 11, 14), presumably by promoting interactions with the cellular machinery that regulates nucleo-cytoplasmic shuttling (10, 14, 25, 42, 43, 48). In addition to nuclear transport, Vpr functions in many processes, including the induction of cell-cycle arrest at the G2

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phase (17), the regulation of apoptosis (2, 3, 35-37), and splicing (24). The numerous biological activities of Vpr appear to be related to its interactions with a variety of cellular partners. Indeed, it has been suggested that importin α binds to Vpr (1, 42, 43, 48) to promote its passage through the NPC (19). We have previously shown that the region between residues 17 and 74 of Vpr, designated N17C74, is a *bona fide* NLS. In addition, Vpr seems to be targeted first to the NPC via interaction with the αH3 region, located between residues 46 and 74, and then enters the nucleus in a process that involves the αH1 region, located between residues 17 and 34 (19). However, it remains to be clarified whether Vpr is transported into the nucleus by importin α alone, without the need for other soluble factors such as importin β.

In this investigation, we have studied the detailed mechanism of Vpr nuclear import and its correlation with HIV-1 infectivity in primary monocytes and macrophages. We demonstrate the following: 1) In digitonin-permeabilized cells, Vpr alone is targeted to the perinuclear region and then transported into the nucleus by importin α in an importin β-independent manner. 2) The three major isoforms of importin α support the apparent nuclear import of Vpr. 3) Primary monocytes exhibit a marked increase in the expression of importin α isoforms upon induction of differentiation to macrophages. 4) The expression of importin α is essential for the nuclear import of Vpr in macrophages. 5) The interaction between importin α and Vpr is indispensable, not only for the importin α-mediated nuclear import of Vpr, but also for the replication of HIV-1 in primary macrophages.

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MATERIALS AND METHODS

Cell culture, cytoplasmic extract preparation, and RNA extraction. Human cervical HeLa cells and African green monkey COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. Human PBMC were isolated on a Ficoll (lymphosepal; IBL) gradient from a healthy HIV-1 seronegative donor. Monocytes were selected from the PBMC by a magnetic cell-separation system with microbeads coated with a CD14-specific monoclonal antibody (MAb), according to the manufacturer's instructions (MACS system; Miltenyi Biotech). Monocytes were plated at the desired density onto 5-mm diameter poly-L-lysine-coated glass-bottomed microwell dishes (Matek Corp.) and grown in RPMI medium (Invitrogen) containing 10% heat-inactivated fetal calf serum (FCS), 5% human serum, and 20 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech) for 1 week, until they spontaneously differentiated into mature macrophages (34).

Cells were lysed in cold, hypotonic buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.3 M sucrose, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cytoplasmic extracts were clarified by centrifugation at 8,000 x *g* for 30 sec and the supernatants were subjected to Western blotting analysis and an *in vitro* nuclear transport assay.

Total cellular RNAs were isolated from monocytes and differentiated primary macrophages using TRIzol reagent (Invitrogen).

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Plasmid constructions. Construction of the glutathione S-transferase (GST)- and green fluorescent protein (GFP)-tagged mutant forms of Vpr, N17C74-GFP and GST-αLA/N17C74-GFP, was as previously described (15, 19). For construction of GST-αH1-GFP and GST-αLA/αH1-GFP, fragments encoding each sequence were prepared by digestion with *Eco*R V and *Not* I, from pGFP-FαH1 and pGFP-FαLA/αH1 (18), respectively. Each fragment was subcloned into *Bam*H I/*Not* I-digested pGEX-6P3 (GE Healthcare). The *Bam*H I site was blunted with KOD DNA polymerase (Toyobo) for ligation with an *Eco*R V site. The GST-tagged human importin α1, α3, and α5 isoforms were constructed as follows: Insert fragments were isolated from pGEX-2T/importin α1, pGEX-2T/importin α5, and pGEX-2T/importin α3, and subcloned into pGEX-6P3 (30). This vector includes the GST coding region and a flag tag at the N- and C-termini of the multi-cloning site, respectively. For the importin α1-deletion mutant that lacked the IBB domain, the ΔIBB importin α fragment was amplified with the primers 5'-TATGGATCCAGCTCCTTTCCTGAT-3' and 5'-GGCCTCGAGGTAAAAGTTAAAGGTCCCAGG-3', using pGEX-6P3/importin α1 as a template. GST- and HA-tagged human importin β was cloned into pGEX-2T at the *Bam*H I and *Kpn* I sites. This fragment was then subcloned into pGEX-6P3 at the *Bam*H I and *Xho* I sites. The GST-tagged SV40-NLS-GFP construct was as previously described (19). An infectious molecular clone, HIV-1 pNF462, a clone that encoded the Vpr-negative ATG-mutant (ΔVpr) and a clone that encoded substitution mutant of Vpr designated αLA, were as previously described (15).

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Expression and purification of recombinant proteins. GST-, and GFP-tagged mutant forms of Vpr and GST-tagged importin α1, α3, and α5 and importin β were expressed in the *Escherichia coli* strains NovaBlue (Novagen) or BL21 CodonPlus (DE3)-RIL (Stratagene) and purified as described elsewhere (15, 19). GST-tagged SV40-NLS-GFP (18) and Ran/TC4 (28) were also expressed in *E. coli* and purified as described (15, 19).

Western blotting. Cell lysates were examined by immunoblotting with MAb against importin α1 (BD Biosciences), MAb against importin α3 (MBL), MAb against importin α5/7 (MBL), MAb against importin β (BD Biosciences), MAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; BD Biosciences), anti-HIV-1 Vpr rabbit serum (NIH AIDS Research and Reference Reagent Program), or MAb against HIV-1 Gag (p24) (15), followed by horseradish peroxidase-linked sheep antibodies against mouse or rabbit IgG (GE Healthcare), as previously described (37).

Quantitative real-time PCR. Approximately 600 ng total RNA were used for the reverse-transcription (RT) reaction, which was performed with the Superscript Preamplification System (Invitrogen). The RT product was used for real-time quantitative PCR of three importin α isoforms in the LightCycler System (Roche) in the presence of LightCycler-FastStart DNA Master SYBR Green I (Roche), using importin α1, α3, and α5 isoform-specific primers as follows: forward α1, 5'-GCATAATAGAACCGTTGATG-3', reverse α1, 5'- AGGAGCCCCATCCTGAAC-3'; forward α3, 5'-AGTGGCTTACCTTATCCAAC-3', reverse α3,

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5'-TGTTGGTACATTGGCAGATG-3'; forward α5, 5'-GTGATCTCCTCACGGTCATG-3', reverse α5, 5'-CATAGGAGCCTCACACTG-3'. Hypoxanthine phosphoribosyltransferase (HPRT) cDNA was amplified as a normalization control, using the primers forward HPRT, 5'-GCCCTGGCGTCGTGATTAGT-3', and reverse HPRT, 5'-GCTCTACTAAGCAGATGGCC-3'.

Microinjection and imaging analysis. GST and GFP fusion proteins (1 mg/ml) were injected into the cytoplasm of primary macrophages grown on a 35-mm glass-bottomed dish with an InjectMan N12 microinjector (Eppendorf). Images were captured with a 40X long-distance objective lens on an Olympus IX70 inverted microscope equipped with a YOKOGAWA CSU10 confocal laser-scanning system controlled by Metamorph software (Universal Imaging).

Small interfering RNA (siRNA) transfection. siRNAs corresponding to importins α1, α3, α5, and β were designed from BLOCK-iT RNAi Designer (Invitrgen) and obtained. The siRNA sequences targeting importins α1, α3, α5 and β were CCAAGCUACUCAAGCUGCCAGGAAA; for α1, CAGUGAUCGAAAUCCACCAAUUGAU; for α3, CCGGAAUGCAGUAUGGGCUUUGUCU; for α 5, CAGUCUGGCUGAAGCUGCUUAUGAA and CACAGCACUGCAGUCUGGAUUCCUU; for β. HeLa cells were seeded 1 day before transfection with siRNAs. At day 0, transfection of importins α1, α3, α5 and β specific

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siRNAs, hypoxantine guanine phosphoribosyltransferase 1(HPRT-S1) specific siRNA as a positive control, or a nonspecific siRNA as a negative control were carried out with Lipofectamine RNAiMAX (Invitrogen) following the company protocol. After 2days, second transfection was performed. After another 2 days, cells were harvested and cytoplasmic extracts were prepared. Transfection efficiency of siRNA was determined by using Western blotting with MAbs against importin α1, α3, α5/7 and β.

HIV-1 infection with macrophages. HIV-1 was introduced into COS cells by electroporation of macrophage-tropic pNF462 viruses encoding wild type Vpr, a mutant form (αLA), and a Vpr-deficient form. Viral supernatants were harvested 72 h after transfection. Virus stocks were titered by measuring the amount of p24 antigen in the culture supernatants using an enzyme-linked immunosorbent assay (ELISA). Differentiated primary macrophages seeded onto a 48-well tissue-culture plate (2 x 10^5/well) were exposed to virus containing 5 ng of p24 antigen for 2 h at 37°C. They were then washed three times and the infected cells were maintained in RPMI 1640 containing 10% FCS, 5% human serum, and M-CSF (20 ng/ml). Culture supernatants were harvested at 3- or 4-day intervals and viral production was monitored by the sequential quantification of p24 antigen in cell-free supernatants with an HIV-1 $p24^{gag}$ ELISA kit (ZeptoMetrix Corp.).

Other assays. The *in vitro* nuclear transport and *in vitro* pull-down assays were performed as previously described (15, 19).

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RESULTS

The nuclear import of Vpr is promoted by importin α without a requirement for importin β. We previously reported that Vpr is localized at the nuclear envelope and that it then traverses the NPC in an importin α-dependent manner (19). To determine whether the human importin α-driven nuclear import of Vpr requires importin β, we reconstituted the candidate factors required for Vpr nuclear transport from digitonin-permeabilized semi-intact HeLa cells. For this experiment we used the region between residues 17 and 74 (N17C74) of Vpr, because this is a functionally transportable region (18, 19). The chimeric protein of N17C74 fused at the N-terminus to GST and at the C-terminus to GFP (~63 kDa) was larger than the limitation for passive diffusion into the nucleus. As shown in Fig. 1, N17C74 localized predominantly to the peri-nuclear region in the absence of soluble factors. Interestingly, importin α1 alone had the highest activity for the nuclear import of N17C74, whereas the addition of importin β decreased the efficiency of N17C74 import, in the presence or absence of RanGDP (Fig. 1, upper panels). These results contrast with the classical nuclear import of SV40-NLS, which requires importin α1, importin β, and RanGDP (Fig. 1, lower panels), but are in good agreement with our recent report (19). Next, to exclude the possibility that residual endogenous importin β in the permeabilized cells contributed to the nuclear migration of Vpr, an importin α1 mutant (ΔIBB), lacking the IBB domain and unable to bind to importin β, was used instead of the full-length importin α1. ΔIBB importin α1 alone enhanced the nuclear import of N17C74, indicating that Vpr can

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enter the nucleus in an importin β-independent manner.

These results support the notion that Vpr is targeted to the peri-nuclear region and then transported into the nucleus by importin α1 alone, without importin β. Thus, the mechanism of nuclear entry of Vpr is quite different from that mediated by the classical transport system.

The nuclear import of Vpr is promoted by all three major isoforms of importin α. In human cells, at least six importin α isoforms have been identified and the isoforms are believed to differ in their efficiency with respect to classical substrate-specific import (31, 45). To determine whether the other two major importin α isoforms, α3 and α5, promote the nuclear import of Vpr similarly to α1, we performed an *in vitro* transport assay using recombinant human α1, α3, and α5. As shown in Fig. 2A, N17C74 was imported into the nucleus by either importin α3 or α5 as well as by α1, suggesting that Vpr is able to utilize all three major importin α isoforms for nuclear entry. The classical nuclear import of SV40-NLS, the positive control for the *in vitro* transport assay, occurred in the presence of any of the three importin α isoforms in combination with importin β and RanGDP (data not shown).

To further examine whether N17C74 directly interacts with the three importin α isoforms, recombinant importin α1, α3, and α5 were incubated with GST- and GFP-tagged N17C74 that was immobilized on glutathione-Sepharose (Fig. 2B). Although the three isoforms share only approximately 50% overall amino-acid

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sequence similarity, the tagged N17C74 protein was able to interact with all of them. These results suggest that the three importin α isoforms can directly interact with Vpr and support its nuclear entry.

Importin α in differentiated macrophages promotes the nuclear import of Vpr. Vpr has been implicated as playing an important role in the nuclear import of proviral DNA in monocyte-derived macrophages (7, 11, 14). Neil *et al.* reported that, although HIV-1 PIC entered the nucleus of primary monocyte-derived macrophages, active nuclear import of PIC was inefficient in monocytes (34). These results suggested that the efficiency of HIV-1 PIC nuclear import in monocytes and macrophages might correlate with that of Vpr. However, it was unclear whether importin α was expressed in monocytes and macrophages, interacted with Vpr, and contributed to HIV-1 replication. Therefore, we first investigated the mRNA expression level of each importin α isoform in primary monocytes and differentiated macrophages. Primary monocytes were prepared from PBMC of normal healthy donors and allowed to differentiate into macrophages *in vitro* in the presence of M-CSF. Total cellular RNA was isolated from primary monocytes and macrophages, and real-time quantitative RT-PCR was conducted using specific primers for each isoform (Fig. 3A). Differentiated macrophages showed higher levels of expression of all three isoforms than did the monocytes. Differences in the protein levels of each importin α isoform between

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monocytes and macrophages were confirmed by Western blotting with MAbs against importin α1, α3, and α5 (Fig. 3B). These results were consistent with the observed levels of mRNA expression. These data clearly show that the expression of the three major isoforms of importin α is low in primary monocytes, but is markedly increased by inducing differentiation into macrophages.

Next, we performed an *in vitro* nuclear transport assay using cytoplasmic extracts from monocytes and differentiated macrophages (Fig. 3C). As expected from the above results, N17C74 was efficiently transported into the nucleus when a cytoplasmic extract from differentiated macrophages was added, whereas the nuclear import of N17C74 was inefficient when a monocyte extract was used. Under these conditions, the classical nuclear import of SV40-NLS was also inefficient with monocyte extracts and efficient with macrophage extracts. These results suggest that the efficiency of nuclear transport of Vpr and SV40-NLS depends strongly on the expression level of importin α in monocytes and macrophages, which are important targets for HIV-1 infection.

Importin α depletion prevents the nuclear import of Vpr in macrophages. To further investigate the requirement for importin α in the nuclear import of Vpr in primary macrophages, cytoplasmic extracts were depleted using a MAb against importin α1 and protein A-Sepharose, prior to the *in vitro* nuclear transport assay. Western blotting analysis of depleted lysates unexpectedly demonstrated that most importin α, including importins α3 and α5 as well as importin α1, were successfully

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removed, whereas importin β remained in the extracts (Fig. 4A). The addition of these extracts drastically decreased the nuclear import of N17C74, compared to that observed with cytoplasmic extracts incubated with preimmune normal mouse IgG (Fig. 4B). Similar results were obtained with SV40-NLS as a control for classical import. In addition, we successfully knocked-down the three importin α isoforms α1, α3 and α5 in HeLa cells by isoform-specific siRNA transfection (Fig. 5A). The nuclear import of N17C74, which was enhanced by cytoplasmic extract of HeLa cells, was greatly decreased by cytoplasmic extract from importin α-siRNA-transfected cells, but not from importin β-, HPRT-, or negative control-siRNA-transfected cells (Fig. 5B).

These results suggest that endogenously expressed importin α is essential for the efficient nuclear import of Vpr.

Interaction of importin α with the αH1 domain of Vpr is essential for its nuclear import in macrophages. To understand the mechanism of the importin α-driven nuclear import of Vpr in detail, we examined the effect of mutations on the nuclear import of Vpr. Vpr consists of three α-helical domains (αH1, αH2, and αH3; Fig. 6A). As we showed previously, N17C74 interacts directly with importin α1 through the αH1 and αH3 domains, and the interaction via αH1 is essential for nuclear entry (19). Therefore, in this study we focused on the αH1-domain interaction. Three mutants were used in this study: αLA/N17C74, in which the Leu residues at positions 20, 22, 23, and 26 within αH1 of Vpr were replaced by Ala residues; the αH1

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domain alone; and its mutant αLA/αH1 (Fig. 6A). In pull-down assays, we found that the αH1-chimeric protein interacted strongly with endogenous importin α in the cytoplasmic extract of HeLa cells, whereas the αLA/αH1 protein interacted very poorly. A similar result was obtained with recombinant importin α (Fig. 6B). These results indicate that the Leu residues at positions 20, 22, 23, and 26 in the αH1 region of Vpr are crucial for binding to importin α.

To further demonstrate the importance of this region for the nuclear transport of Vpr, we carried out an *in vitro* nuclear transport assay with the αLA/N17C74 chimeric protein using digitonin-permeabilized HeLa cells. As shown in Fig. 6C, the αLA/N17C74 mutant had completely lost the ability to migrate into the nucleus in the presence of cytoplasmic extract prepared from primary macrophages, even in the presence of recombinant importin α. The localization of N17C74 and of the αLA/N17C74 mutant protein was determined after cytoplasmic microinjection into primary macrophages. N17C74 clearly localized in the nucleus, whereas the mutant did not, suggesting that the mutant had lost nuclear-import activity (Fig. 6D). These findings clearly indicate that the ability of Vpr to interact with importin α is indispensable for its nuclear import in macrophages.

Interaction of importin α through the αH1 domain of Vpr is crucial for the efficient replication of HIV-1 in macrophages. Finally, it was important to determine whether the nuclear entry of Vpr mediated by its interaction with importin α, is crucial for macrophage-tropic HIV-1 replication in primary macrophages. We compared the

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replication in primary macrophages of HIV-1 encoding wild type Vpr, a Vpr-negative ATG mutant (ΔVpr), and a mutant form (αLA) that cannot interact with importin α and thus is defective in nuclear transport. The αLA mutant retained the ability to induce G2 arrest and apoptosis (data not shown). To examine whether the αLA-mutant virus synthesized mutant Vpr protein, HeLa cells was transfected with pNF462 proviral DNA encoding wild type Vpr or the Vpr-mutant form (αLA) and the cell lysates were analyzed by Western blotting using anti-Vpr antibody and anti-Gag antibody. The wild type and αLA-mutant transfectants had similar amounts of Vpr and $p24^{Gag}$ (Fig. 7A), suggesting that the wild type and mutant proteins were similarly incorporated into the virus. Typical kinetics for the replication of these wild type and mutant viruses in the primary macrophages of two donors are shown in Fig. 7B. The virus encoding wild type Vpr replicated well in both donors, at viral inputs of 5 ng p24 antigen, and replication reached a peak 14~17 days after infection. In contrast, ΔVpr-HIV-1 displayed a markedly decreased level of replication as compared to the wild type virus, suggesting the importance of Vpr in HIV-1 replication in primary macrophages. Interestingly, replication of the αLA-mutant virus was also reduced to a level equivalent to that of the ΔVpr virus. Similar results where obtained with macrophages isolated from four additional donors (data not shown).

Our results strongly support the notion that the binding of Vpr to importin α is essential for the nuclear import of Vpr, and that this nuclear import is crucial for viral replication in macrophages. In addition, our results indicate that expression of importin

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α is essential for efficient viral replication in macrophages.

DISCUSSION

A novel nuclear import mechanism for Vpr promoted by importin α. We previously showed that Vpr traverses the NPC in an importin α-dependent manner (19). Our present study clearly demonstrates that importin α promotes nuclear import of Vpr in digitonin-permeabilized cells, without the need for importin β or other soluble proteins. The importin α derivative ΔIBB, which cannot bind to importin β, also promotes nuclear import of Vpr. In addition, depletion of three importin α isoforms from HeLa cells by siRNA markedly decreased the nuclear import of Vpr in an *in vitro* nuclear transport assay using cytoplasmic extracts, but not in importin β-depleted extracts. These results strongly suggest that Vpr is transported into the nucleus by importin α alone, without utilizing the classical importin β-dependent transport pathway. To our knowledge, Vpr is the first retroviral protein that has been shown to use a nuclear import mechanism involving importin α alone without any other soluble factors. Moreover, as previously reported (19), Vpr directly localizes to the perinuclear region, without a requirement for any soluble factors, before it is transported into the nucleus by importin α. This perinuclear localization distinguishes the nuclear import of Vpr from that of other NLS-bearing proteins, suggesting that the karyophilic properties of Vpr rely on a novel mechanism.

Our present and previous results enable us to characterize the nuclear import of Vpr as follows. 1) Vpr is targeted to the perinuclear region in the absence of soluble factors, as shown by *in vitro* transport assays (Fig. 1; 19). 2) The transport of Vpr is mediated by importin α alone, without the intervention of importin β, transportin,

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